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Functions of FKBP12 and Mitochondrial Cyclophilin Active Site Residues In Vitro and In Vivo in *Saccharomyces cerevisiae*

Kara Dolinski,* Christian Scholz,† R. Scott Muir,‡ Sabine Rospert,§ Franz X. Schmid,† Maria E. Cardenas,* and Joseph Heitman*‡‖

Departments of *Genetics and ‖Pharmacology, and the ‡Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710; †Biochemisches Laboratorium, Universität Bayreuth, D-95440 Bayreuth, Germany; and §Department of Biochemistry, Biozentrum, Basel, Switzerland CH-4056

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Cyclophilin and FK506 binding protein (FKBP) accelerate *cis–trans* peptidyl-prolyl isomerization and bind to and mediate the effects of the immunosuppressants cyclosporin A and FK506. The normal cellular functions of these proteins, however, are unknown. We altered the active sites of FKBP12 and mitochondrial cyclophilin from the yeast *Saccharomyces cerevisiae* by introducing mutations previously reported to inactivate these enzymes. Surprisingly, most of these mutant enzymes were biologically active in vivo. In accord with previous reports, all of the mutant enzymes had little or no detectable prolyl isomerase activity in the standard peptide substrate-chymotrypsin coupled in vitro assay. However, in a variation of this assay in which the protease is omitted, the mutant enzymes exhibited substantial levels of prolyl isomerase activity (5–20% of wild-type), revealing that these mutations confer sensitivity to protease digestion and that the classic in vitro assay for prolyl isomerase activity may be misleading. In addition, the mutant enzymes exhibited near wild-type activity with two protein substrates, dihydrofolate reductase and ribonuclease T1, whose folding is accelerated by prolyl isomerases. Thus, a number of cyclophilin and FKBP12 "active-site" mutants previously identified are largely active but protease sensitive, in accord with our findings that these mutants display wild-type functions in vivo. One mitochondrial cyclophilin mutant (R73A), and also the wild-type human FKBP12 enzyme, catalyze protein folding in vitro but lack biological activity in vivo in yeast. Our findings provide evidence that both prolyl isomerase activity and other structural features are linked to FKBP and cyclophilin in vivo functions and suggest caution in the use of these active-site mutations to study FKBP and cyclophilin functions.

INTRODUCTION

Anfinsen's classic studies established that protein primary amino acid sequences can be sufficient to direct folding into tertiary structures (Anfinsen, 1973). During the last several years, two types of proteins have been discovered that facilitate protein folding both in vitro and in vivo (Gething and Sambrook, 1992). Chaperones bind to folding proteins and inhibit aggregation of folding intermediates. In addition, enzymes have been discovered that accelerate rate-limiting steps during protein folding in vitro. Examples include protein disulfide isomerase and peptidyl-prolyl isomerase. Protein disulfide isomerase catalyzes reactions between sulfhydryl groups, accelerating the process by which correct disulfide bonds are formed. Prolyl isomerases, which include cyclophilins

‖ Corresponding author: 322 Carl Building, Research Drive, Box 3546, Duke University Medical Center, Durham, NC 27710.

and FKBPs, catalyze *cis–trans* peptidyl-prolyl isomerization, are highly conserved from bacteria and yeast to humans, and are found in multiple cellular compartments (Dolinski and Heitman, 1997). These features suggested prolyl isomerases might play a general essential role in protein folding. This role would be distinct from the well-established roles of cyclophilin A and FKBP12 in mediating toxic and immunosuppressive effects of the natural products cyclosporin A (CsA), FK506, and rapamycin, which involve inhibition of calcineurin and TOR functions by immunophilin–drug complexes (Heitman *et al.*, 1992; Schreiber and Crabtree, 1992; Schmid, 1993; Schmid *et al*., 1993; Fischer, 1994).

Although the enzymatic and immunosuppressive activities of the immunophilins have been known for quite some time, their biological functions are largely unknown. It has been widely assumed that the prolyl isomerase activity of the cyclophilins and FKBPs is relevant to in vivo function given that 1) isomerase activity is conserved in diverse cyclophilins and FKBPs (Gething and Sambrook, 1992; Heitman *et al.*, 1992; Fischer, 1994), 2) cyclophilins and FKBPs catalyze rate-limiting steps during protein refolding in vitro (Kiefhaber *et al.*, 1990a; Tropschug *et al.*, 1990; Schonbrunner *et al.*, 1991), and 3) immunosuppressants bind to the isomerase active sites, potently inhibiting enzyme activity and, in some cases, impairing de novo protein folding (Lodish and Kong, 1991; Steinmann *et al.*, 1991; Stein, 1993; Matouschek *et al.*, 1995; Rassow *et al.*, 1995; Rospert *et al.*, 1996). Cyclophilins and FKBPs are nonessential, however, and have few subtle mutant phenotypes in microorganisms, arguing against an essential general role in protein folding (Heitman *et al.*, 1991a,b; Davis *et al.*, 1992; Nielsen *et al.*, 1992; Frigerio and Pelham, 1993; Manning-Krieg *et al.*, 1994). In addition, cyclophilins and FKBPs are abundant proteins, expressed at levels higher than might be expected of enzymes. Furthermore, because spontaneous prolyl isomerization is accelerated in hydrophobic solvents (Eberhardt *et al.*, 1992), binding in a hydrophobic protein environment could contribute to catalysis (Schreiber and Crabtree, 1992).

Two recent studies suggest that the enzymatic activity of cyclophilins may be dispensible for their physiological functions. The first notable example is the *Drosophila* cyclophilin homologue, ninaA, an endoplasmic reticulum membrane anchored protein required for proper maturation of rhodopsins (Schneuwly *et al.*, 1989; Shieh *et al.*, 1989; Colley *et al.*, 1991; Stamnes *et al.*, 1991). In ninaA mutant flies, rhodopsins misfold and accumulate in the endoplasmic reticulum, resulting in visual system defects. NinaA forms a stable complex with rhodopsin, and subtle decreases in ninaA levels significantly impair function (Baker *et al.*, 1994). Thus, ninaA may act stoichiometrically rather than catalytically (Baker *et al.*, 1994).

A second well-studied example involves the complex between human cyclophilin A and the human immunodeficiency virus 1 (HIV-1) GAG protein (Luban *et al.*, 1993), which enables cyclophilin A to be packaged into HIV virions (Franke *et al.*, 1994). CsA disrupts the cyclophilin A–GAG interaction, resulting in the production of HIV virions lacking cyclophilin A. The resulting virions have defects at an early stage after viral entry into the infected cell (Braaten *et al.*, 1996b). Mutation of the cyclophilin A binding pocket/active site prevent GAG binding (Braaten *et al.*, 1997), and the recent solution of the x-ray crystal structure of a cyclophilin A–GAG complex reveals a proline-rich turn bound in the cyclophilin A active site with the peptidyl-prolyl bond in a *trans* configuration (Gamble *et al.*, 1996; Zhao *et al.*, 1997). This region of GAG had been previously implicated in cyclophilin binding and was found to be mutated in CsA-resistant cyclophilin-independent HIV mutants (Aberham *et al.*, 1996; Braaten *et al.*, 1996a). Thus, these studies have led to a model in which cyclophilin A serves a structural rather than a catalytic role for HIV function (Luban, 1996).

Other studies, however, have suggested that other prolyl isomerases do require enzymatic activity in vivo. Studies of the human prolyl isomerase PIN1, a member of the parvulin family (Lu *et al.*, 1996), have been interpreted to suggest that prolyl isomerase activity is the essential function of PIN1. ESS1, the yeast homologue of PIN1, is essential, and expression of human PIN1 restores viability in yeast *ess1* mutant strains (Lu *et al.*, 1996). Because a PIN1 mutant altered in three conserved C-terminal residues lacked prolyl isomerase activity in vitro and failed to complement *ess1* in vivo, it was concluded that prolyl isomerase activity is the essential PIN1 function (Lu *et al.*, 1996). These mutations, however, could perturb some other essential feature of PIN1, such as protein binding or conformational stability, and further study will be necessary to establish this point. Another example is the recently identified cyclophilin RanBP2 that is involved in the biogenesis of retinal opsins (Ferreira *et al.*, 1996). Active-site mutations prevent binding and alteration of opsin by the RanBP2 cyclophilin, and these findings were interpreted to suggest the function of this interaction is prolyl isomerization of opsin by RanBP2 cyclophilin (Ferreira *et al.*, 1996).

Our studies have investigated in detail the prolyl isomerase activity of FKBP12 and mitochondrial cyclophilin in yeast and its link to biological function. We found that several previously reported active site mutants (Aldape *et al.*, 1992; Zydowsky *et al.*, 1992; Timerman *et al.*, 1995; Ferreira *et al.*, 1996) of both cyclophilin and FKBP12 were fully functional in vivo. Although these mutant enzymes do lack activity in the

standard prolyl isomerase assay, which is based on isomer-specific cleavage of a tetrapeptide by chymotrypsin, we discovered that this is a secondary consequence of increased protease sensitivity of the mutant enzymes. Indeed, these mutant enzymes exhibit readily detectable activity in a protease-free peptide folding assay, and near wild-type activity with two different protein substrates (dihydrofolate reductase [DHFR] and ribonuclease T1) in the absence of protease. Our studies provide evidence that prolyl isomerase activity and other structural features of mitochondrial cyclophilin and FKBP12 are linked to in vivo function and suggest caution in the use of these mutations to determine in vivo functions of cyclophilin and FKBP prolyl isomerase activity.

MATERIALS AND METHODS

Site-directed Mutagenesis of Yeast FKBP12 and Mitochondrial Cyclophilin

The R73A and H144Q mutations in cyclophilin Cpr3 are analogous to R55A and H126Q in human cyclophilin A (Zydowsky *et al.*, 1992) and were engineered by polymerase chain reaction (PCR) overlap mutagenesis as described (Ho *et al.*, 1989) by using the following mutagenic primers: 1) R73A (5′-CTTTCCACGCAATCATCCCAGACTTC-3′ and 5′-GGGATGATTGCGTGGAAAGGGACACC-3′) and 2) H144Q (5′-GATGGAAAACAGTGGGTCTTTGGTGAG-3′ and 5′-AAAGACAACCTGTTTTCCATCCAACC-3′).

Flanking primers for the R73A and H144Q mutations were 5′-CCT-T**GGATCC**AATAACCAATAAGAATTATTTTAGTCC-3′ (where the *Bam*HI site is in boldface type) and 5′-ATAT**AAGCTT**AAAAGTTG-GTTGATTTTTTATGAGCC-3′ (where the *Hin*dIII site is in boldface type). PCR products were cleaved with *Bam*HI/*Hin*dIII and cloned in the *CEN URA3* plasmid pRS316 (Sikorski and Hieter, 1989).

The D44V and F106Y mutations in yeast FKBP12 were engineered by PCR overlap mutagenesis (Ho *et al.*, 1989) as described for the F43Y mutation (Lorenz *et al.*, 1995) with the following mutagenic primers: 1) D44V (5′-CCAAAAATTCGTCTCCTCCGTTGACAGG-3′ and 5′-CA-ACGGAGGAGACGAATTTTTGGCCGTTC-3′) and 2) F106Y (5′-CTT-TGGTTTACGACGTCGAATTGTTG-3′ and 5′-ACAATTCGACGTC-GTAAACCAAAGTACTGTTTGG-3′). Flanking primers were 5′-CG-GGTTAGATGATATCCCACAG-3′ and 5′-GGAATTCATAAGCATT-TCCACATG-3′. The resulting PCR products were cleaved with *Eco*RI, cloned in *CEN LEU2* plasmid YCplac111 (Gietz and Sugino, 1988), and expressed in FKBP12 mutants JHY2–1c or CHY516.

Epitope Tagging CPR3 and Mitochondrial Fractionation

The wild-type and mutant *CPR3* genes were amplified from the plasmids described above using the following primers: 5′-ATATAT-GGATCCTCTACTTACCATGTTTAAACG-3′ (where the *Bam*HI site is in boldface type) and 5′-GCGCTATA**GCGGCCGC**GTAACT-CACCAGCTTCTTCGAT-3′ (where the *Not*I site is in boldface type). PCR products were gel purified, digested with *Bam*HI/*Not*I, and cloned into the *Bam*HI/*Not*I sites of pYeF2 to hemagglutinin-epitope tag the CPR3 protein at the carboxyl terminus (Cullin and Minvielle-Sebastia, 1994). Total cell extracts and mitochondrial fractions were prepared as described (Yaffe, 1991).

Protein Purification

The genes encoding the F43Y, D44V, and F106Y FKBP12 mutants described above were PCR amplified, cloned in bacterial His6 expression plasmids pV2a or pTrcHisB (Invitrogen, San Diego, CA), as described for wild-type yeast FKBP12 (Cardenas *et al.*, 1994). The wild-type and R73A and H144Q mutant *CPR3* genes from yeast expression plasmids described above were PCR amplified with primers: 5′-TATATA**GGATCC**GGGTAAAAAAGTGTTCTTTGATCC-3′ (where the *Bam*HI site is in boldface type) and 5′-ATAT**AAGCTT**AAAAGT-TGGTTGATTTTTTATGAGCC-3′ (where the *Hin*dIII site is in boldface type). PCR products were gel purified, digested with *Bam*HI/*Hin*dIII, and ligated in pTrcHisB (Invitrogen). The resulting His6-Cpr3 proteins lack the mitochondrial leader sequence. All plasmids were confirmed by DNA sequencing. The wild-type and mutant FKBP12 and mitochondrial cyclophilin proteins were purified by Ni^{2+}-affinity chromatography (Cardenas *et al.*, 1994; Heitman *et al.*, 1993).

Measurements of Prolyl Isomerase Activity in the Peptide Assays

In both the protease-coupled and the protease-free assay the chromogenic peptide succinyl-Ala-Phe-Pro-Phe-4-nitroanilide was used as the substrate. The traditional coupled assay with chymotrypsin (Fischer *et al.*, 1984) was performed as described in detail by Scholz *et al.* (1997b). In all experiments the FKBP12 proteins were coincubated with the protease for 5 min before the peptide was added to initiate the assay reaction. The *cis–trans* isomerization of the Phe-Pro imide bond, coupled with the chymotryptic cleavage of the *trans* peptide, was followed by the increase in absorbance at 390 nm of the liberated 4-nitroaniline in a HP 8452 diode array spectrophotometer. The peptide concentration in the protease-linked assay was 78 μM. Monoexponential functions were fit to the progress curves and the activity was calculated from the observed rate constants (Fischer *et al.*, 1984).

In the protease-free assay the *cis–trans* isomerization of the Phe-Pro peptide bond in the intact assay peptide is followed by the small decrease of the absorbance at 330 nm of the uncleaved nitroanilide moiety (G. Fischer, personal communication). In the spectrophotometer cell, 955 μl of 0.1 M Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, and 30 μl of the FKBP12 variant were mixed and incubated for 10 min to allow thermal equilibration. The assay was initiated by adding 15 μl of the assay peptide (7.8 mM in trifluoroethanol/0.45 M LiCl). During the assay the *trans:cis* equilibrium changes from 60:40 to 90:10. Peptide concentration in the protease-free assay was 120 μM.

Isolation of Mitochondria and Preparation of Radiolabeled Denatured Su9-DHFR

Yeast cells were cultured in synthetic lactate medium at 30°C. Nycodenz-gradient–purified mitochondria (Glick and Pon, 1995) were isolated from strain JHY80–2B expressing wild-type CPR3 or the CPR3 H144Q mutant protein. Su9-DHFR consists of the first 69 residues of subunit 9 of the *Neurospora crassa* F_1F_0-ATPase fused to mouse DHFR (Pfanner *et al.*, 1987). Synthesis of [^{35}S]methionine-labeled Su9-DHFR was carried out in a reticulocyte lysate (Stueber *et al.*, 1984). After translation, the lysate was depleted of ATP by incubation with 40 U/ml apyrase for 5 min at 30°C. Ribosomes were removed by centrifugation for 15 min at 100,000 $\times$ *g*, the precursor protein was precipitated with 67% ammonium sulfate for 30 min on ice, and precipitated protein was collected by centrifugation for 10 min at 15,000 $\times$ *g*. To obtain denatured Su9-DHFR the pellet was resuspended in 8 M urea, 20 mM Tris-HCl (pH 7.5), and 20 mM dithiothreitol, and incubated for 15 min at room temperature.

DHFR Folding Assay

The folding kinetics of Su9-DHFR in intact mitochondria were determined essentially as described (Matouschek *et al.*, 1995). In the absence of ATP, Su9-DHFR accumulates as unfolded "ATP-depletion intermediate" in the mitochondrial intermembrane space (Manning-Krieg *et al.*, 1991). After another addition of ATP, this intermediate rapidly imports into the matrix space where it folds into a

protease-resistant conformation. To deplete intramitochondrial ATP, mitochondria corresponding to 1 mg of total protein were incubated at 25°C in 3 ml of import buffer (0.6 M sorbitol, 50 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM methionine, 1 mg/ml fatty acid-free bovine serum albumin) containing 10 U/ml apyrase (grade VIII, Sigma, St. Louis, MO), 2 μg/ml efrapeptin (kind gift from Eli Lilly, Indianapolis, IN), and 5 μg/ml oligomycin. After 5 min, NADH was added to a final concentration of 2 mM and 100 μl of denatured Su9-DHFR was added. The ATP-depletion intermediate was allowed to accumulate for 10 min at 25°C. Unimported precursor was digested with 100 μg/ml trypsin for 25 min on ice. Trypsin inhibitor was added to a final concentration of 200 μg/ml and mitochondria were reisolated by centrifugation at 12,000 $\times$ *g*. The mitochondrial pellet was resuspended in 3 ml of import buffer lacking apyrase but supplemented with 50 μg/ml trypsin inhibitor. The mixture was split, and one half was supplemented with 2.5 μg/ml CsA (Sandoz, Basel, Switzerland) in Tetrahydrofuran (THF) with 0.47 M LiCl and the other was supplemented with solvent alone. Both aliquots were incubated 5 min on ice and then 5 min at 30°C. Chase into the matrix was initiated by addition of 150 μl of chase buffer containing 20 mM ATP and 50 mM α-ketoglutarate. Aliquots of 100 μl were taken before and at various times after addition of chase buffer. The fraction of folded DHFR was determined by diluting the aliquots with 100 μl of ice-cold 20 mM HEPES-KOH, pH 7.4, containing 1% Triton X100 and 200 μg/ml proteinase K. After 10 min on ice, proteinase K was inactivated by addition of 1 mM phenylmethylsulfonyl fluoride, protein was precipitated by addition of 5% trichloroacetic acid and the samples were analyzed by SDS-PAGE. To determine the total amount of Su9-DHFR that had reached the matrix space (total import), a sample was taken after the last time point and trichloroacetic acid-precipitated without proteinase K. Folding was determined by fluorography of dried gels and quantified with a Molecular Dynamics model 300A densitometer. The fraction of folded Su9-DHFR is given as percentage of Su9-DHFR present in the matrix (total import). The kinetics of folding were analyzed by assuming a first-order process. Folding half-times (t) were calculated from the rate constants by using the equation $t = \ln 2/k$ (Matouschek *et al.*, 1995).

Prolyl Isomerase Activity Measured in a Protein Folding Reaction

RCM-(S54G/P55N)-RNase T1 was used as a substrate in these assays. (S54G,P55N)-RNase T1 was purified and carboxymethylated as described (Mücke and Schmid, 1993, 1994). The concentrations of RCM-(S54G/P55N)-RNaseT1 were determined spectrophotometrically by using an absorption coefficient of $\epsilon_{278} = 21{,}060\ M^{-1}\cdot cm^{-1}$ (Takahashi *et al.*, 1970).

Refolding at 15°C was initiated by a 1:40 dilution of the unfolded protein (in 0.1 M Tris-HCl, pH 8.0) to final conditions of 2.0 M NaCl and the desired concentrations of the wild-type protein or the mutant forms of FKBP12 or mitochondrial cyclophilin. The folding reaction was followed by the increase in protein fluorescence at 320 nm (10-nm band width) after excitation at 268 nm (1.5-nm band width). The experimental procedures of Scholz *et al.* (1997b) were used. Under the given conditions slow refolding of RCM-(S54G/P55N)-RNase is a monoexponential process in the absence and in the presence of the catalyst FKBP12 or mitochondrial cyclophilin. The rate constants of folding were determined by using the program Grafit 3.0 (Erithacus Software, Staines, United Kingdom). Fluorescence was measured in a Hitachi F4010 fluorescence spectrophotometer.

Yeast Strains

Two strains used herein are isogenic derivatives of *Saccharomyces cerevisiae* strain JK9–3d (*leu2–3, 112 ura3–52 rme1 trp1 his4 HML*a; Heitman *et al.*, 1991b) with the following changes indicated: JHY2–1c, *MAT***a** Δ*ade2 fpr1::ADE2*; JHY80–2b, *MATα cpr3::HIS3*. Strains MB11–3 (Davis *et al.*, 1992) also used were as follows: *MATα ade2–101 trp1-Δ1 ura3–52 his3-Δ200 lys2–801 can1 cpr3::HIS3*; CHY516, *MAT***a** *vph6Δ::TRP1 fpr1::ADE2 ura3–52 his3Δ200 leu2–3,112 trp1Δ101* (Hemenway and Heitman, 1996).

Heterologous Expression of FKBP Domains with Yeast FKBP12 Gene Regulatory Sequences

The human FKBP12 open reading frame was fused to the 5′ and 3′ untranslated regions of yeast FKBP12 by a three-part PCR overlap with the following primers: 1) 5′-AGTTTCAACTTGAACACCCATTATTACTTGTTTTGATTGATT-3′ (792), 2) 5′-AATCAATCAAAACAAGTAATAATGGGTGTTCAAGTTGAAACT-3′ (793), 3) 5′-TCAATTGATAGTACTTTGCAATCATTCCAGTTTCAGGAGCTCAAC-3′ (794), and 4) 5′-GTTGAGCTCCTGAAACTGGAATGAAAGCAAAGTACTATCAATTGA-3′ (795).

Flanking primers were 5′-CGGGTTAGATGATATCCCACAG-3′ (713) and 5′-GGAATTCATAAGCATTTCCACATG-3′ (714). First-round PCR products using primers 713/792, 793/794, and 795/714 were purified and amplified by PCR with flanking primers 713/714. First-round PCR conditions were 3 min at 72°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C; followed by 5 min at 72°C. Second-round PCR conditions were 3 min at 72°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C; followed by 5 min at 72°C. The resulting ~2-kb PCR product was cleaved with *Eco*RI, cloned in YCplac111 (Gietz and Sugino, 1988), and expressed in the *fpr1* mutant strains JHY2–1c and CHY516.

The yeast FKBP13 open reading frame was fused to the 5′ and 3′ untranslated regions of yeast FKBP12 as above with the following primers: 1) 5′-TTCCAAATCTGACAGGGAACCCATTATTACTTGTTTTGATTGATT-3′ (796), 2) 5′-AATCAATCAAAACAAGTAATAATGGGTTCCCTGTCAGATTTGGAA-3′ (797), 3) 5′-TCAATTGATAGTACTTTGCAACTAGGCGGCTGATTTCACGTC-3′ (798), and 4) 5′-GACGTGAAATCAGCCGCCTAGAAGCAAAGTACTATCAATTGA-3′ (799). In this case, first-round PCRs were with primers 713/796, 797/798, and 799/714. PCR products were purified, mixed, amplified with flanking primers, and subcloned as above.

The yeast/human and human/yeast FKBP12 hybrid genes were constructed by cleaving the human or yeast FKBP12 YCplac111 plasmid with *Hpa*I/*Sph*I, purifying the gapped plasmid, and inserting the corresponding *Hpa*I–*Sph*I fragment from the yeast or human FKBP12 gene. All hybrid genes were confirmed by DNA sequencing with primers 5′-GGCCTTTCACCTAAACTCGA-3′ (818) and 5′-TCAGATACTTACCATAAACA-3′ (819) that hybridize to 5′ and 3′ regions common to all of the hybrid genes.

RESULTS

Mitochondrial Cyclophilin "Active-Site" Mutants

To test the role of prolyl isomerase activity in cyclophilin function, we introduced previously described active-site mutations and tested whether the resulting mutants complement a yeast cyclophilin mutation. The yeast mitochondrial cyclophilin Cpr3 is essential for growth at 37°C on medium containing lactate as the sole carbon source because the Cpr3 enzyme is essential for mitochondrial metabolism at elevated temperature and lactate is used via respiration (Davis *et al.*, 1992). On the basis of previous crystallographic and genetic studies of human cyclophilin A (Kallen and Walkinshaw, 1992; Zydowsky *et al.*, 1992; Ke *et al.*, 1993; Mikol *et al.*, 1993; Zhao and Ke, 1996), two point mutations that would be predicted to abolish prolyl isomerase activity, R73A and H144Q, were engineered

into the Cpr3 protein. These correspond to mutations in human cyclophilin A, R55A and H126Q, previously shown to inactivate the human enzyme based on the standard in vitro isomer-specific peptide cleavage assay (Zydowsky *et al.*, 1992).

Cpr3 Active-Site Mutant Cyclophilins Are Inactive in Peptide Cleavage Assay but Catalyze Protein Folding In Vitro

To address the in vitro enzymatic activities of the mutant enzymes, the wild-type and mutant yeast Cpr3 proteins were tagged at the amino terminus of the mature protein (minus the mitochondrial leader sequence) with hexahistidine, expressed in bacteria, and purified via Ni^{2+} affinity chromatography (see MATERIALS AND METHODS). Both the R73A and the H144Q mutant Cpr3 proteins lacked detectable prolyl isomerase activity (with a detection limit of ~0.5% specific activity of the wild-type enzyme) in the in vitro peptidyl-prolyl isomerization assay that involves chymotrypsin cleavage of the *trans* isomer of tetrapeptide substrates Suc-Ala-X-Pro-Phe-*p*-nitroanilide (where Suc is succinyl and X is Ala, Leu, Glu, or Lys) to release the chromogenic group (our unpublished results). In addition, neither the R73A nor the H144Q mutant protein bound appreciably to CsA in an LH-20 drug binding assay (our unpublished results). We conclude that the R73A and H144Q mutations have a similar effect on the prolyl isomerase activity of the yeast mitochondrial cyclophilin and human cyclophilin A, as detected with the in vitro peptide cleavage assay. As will be described in detail below for FKBP12 mutations, and elsewhere for these cyclophilin mutants (Scholz *et al*., 1997b), these active-site mutations result in a loss of activity in the protease-coupled peptide assay because they render the cyclophilin mutant enzymes sensitive to digestion by the chymotrypsin present in the protease-coupled peptide isomerization assay.

In vitro the Cpr3 enzyme catalyzed the prolyl isomerization limited refolding of reduced and *S*-carboxymethylated S54G/P55N ribonuclease T1 (RCM-S54G/P55N-RNase T1) very well (Scholz and Schmid, unpublished results). This protein contains a single *cis* peptidyl-prolyl bond (Tyr^{38}-Pro^{39}). In the unfolded state, 85% of the molecules contain an incorrect trans Tyr^{38}-Pro^{39} bond and refold slowly with a half-time of about 400 s (Kiefhaber *et al.*, 1990a). This refolding reaction is limited in rate by the *trans* to *cis* isomerization of the Tyr^{38}-Pro^{39} peptide bond. The two Cpr3 active site mutant enzymes R73A and H144Q were also active and accelerated the folding of the ribonuclease T1 variant with about two-thirds the efficiency of the wild-type Cpr3 enzyme (Scholz and Schmid, unpublished results). Thus, both the R73A and the H144Q Cpr3 mutant enzymes retain prolyl isomerase activity toward a protein substrate whose folding is rate-limited by a single *trans* to *cis* peptidyl-prolyl isomerization.

Cpr3 Active-Site Mutant Cyclophilin Catalyzes Protein Folding in Mitochondria

To further address whether these Cpr3 active-site mutants retain activity toward another protein substrate, we took advantage of the previous finding that, in conjunction with mitochondrial hsp70, the mitochondrial cyclophilin Cpr3 promotes refolding of a chimeric protein, Su9-DHFR, after import into the mitochondrial matrix (Matouschek *et al.*, 1995; Rassow *et al.*, 1995; Rospert *et al.*, 1996). The Su9-DHFR protein consists of the mouse DHFR protein fused to the mitochondrial matrix targeting sequence of subunit 9 of the F_1F_0-ATPase. In these experiments, radiolabeled Su9-DHFR protein was denatured and incubated with purified mitochondria. After incubation, mitochondria were repurified and lysed with detergent, and the folding state of DHFR was assessed by protease K digestion. In mitochondria expressing the wild-type Cpr3 protein, the half-time of folding was 1.1 min, and ~80% of the imported Su9-DHFR folded into a protease-resistant conformation (Figure 1A and Table 1). In accord with previous reports, addition of CsA inhibited DHFR refolding by about threefold ($t_{1/2}$ = 2.9 min; Figure 1A and Table 1; Matouschek *et al.*, 1995; Rassow *et al.*, 1995). In mitochondria expressing the H144Q Cpr3 mutant protein, the half-time of DHFR folding was only slightly reduced compared with wild-type ($t_{1/2}$ = 1.4 min; Figure 1B and Table 1). The acceleration of DHFR refolding by the H144Q mutant protein was largely insensitive to CsA ($t_{1/2}$ = 1.6 min; Figure 1B and Table 1), consistent with the finding that this mutant does not bind CsA in vitro. In conclusion, the H144Q cyclophilin mutant enzyme retains activity with a protein substrate.

The cyclophilin active-site mutants lack activity in the protease coupled peptide cleavage assay not because they are inactive but rather because they have been rendered protease sensitive by the active-site mutation. This interpretation is supported both by our finding that these mutant enzymes retain near wild-type activity in two different protein folding assays with either ribonuclease T1 or DHFR as substrate (this article), and that these mutants exhibit low but substantial levels of prolyl isomerase activity in a modified peptide assay that avoids coupling with proteolyic cleavage (Scholz *et al*., 1997b).

Mitochondrial Cyclophilin H144Q Mutant Is Functional In Vivo

The genes encoding wild-type and the R73A and H144Q mutant Cpr3 proteins were cloned into low-copy-number centromeric plasmids (MATERIALS

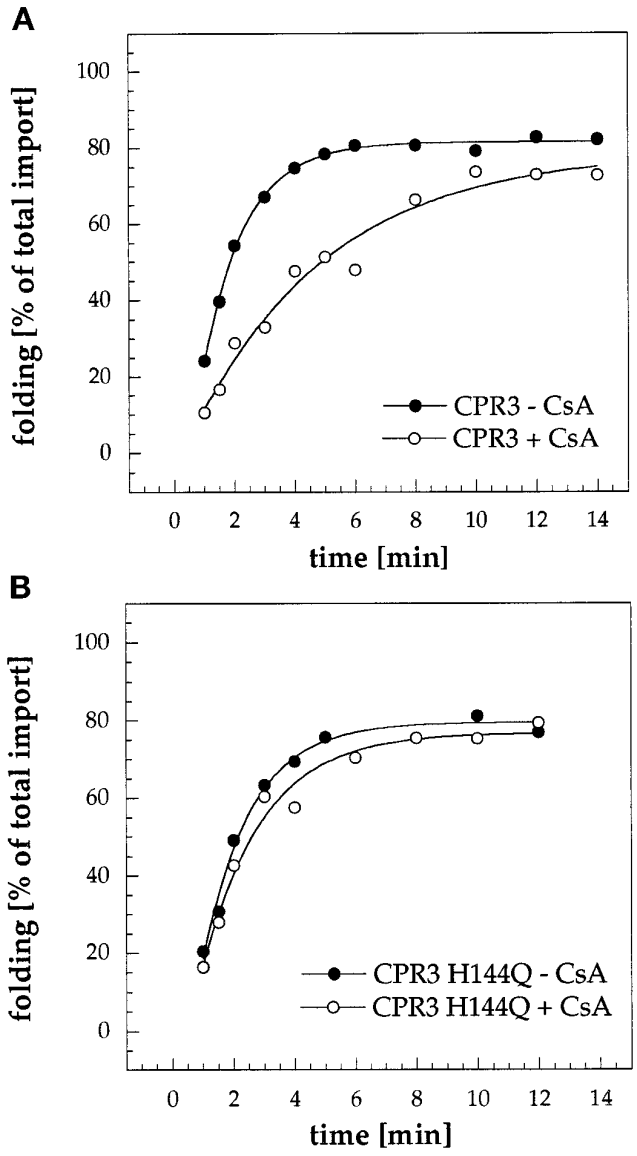

Figure 1. Cpr3 active-site mutant catalyzes protein refolding in mitochondria. Refolding kinetics of Su9-DHFR imported into mitochondria purified from a *cpr3* mutant strain expressing the wild-type CPR3 protein (A) or the CPR3 H144Q prolyl isomerase inactive mutant protein (B). Su9-DHFR was accumulated as the unfolded ATP-depletion intermediate in the mitochondrial intermembrane space. After reisolation, mitochondria were incubated with (+ CsA) or without (− CsA) 2.5 μg/ml CsA. Su9-DHFR was subsequently chased into the matrix by addition of chase buffer containing 2 mM ATP and 5 mM α-ketoglutarate. At the indicated times, samples were withdrawn and treated with Triton X-100 and proteinase K, and the amount of folded protease-resistant DHFR was analyzed by SDS-PAGE and fluorography (for details, see MATERIALS AND METHODS).

AND METHODS) and tested for complementation of the *cpr3* growth defect on lactate medium at 37°C. Wild-type Cpr3 and the H144Q mutant protein complemented and restored growth, whereas the R73A mutant protein did not (Figure 2A). As determined by Western blot analysis, epitope-tagged versions of wild-type Cpr3 and the R73A and the H144Q proteins were expressed at comparable levels (Figure 2B). In addition, Western blot analysis of mitochondrial fractions revealed that the wild-type and both mutant Cpr3 proteins cofractionated with the mitochondrial protein, cytochrome *c* oxidase (COX2; Figure 2B). Thus, the failure of the R73A mutant protein to complement is not attributable to instability or mislocalization and may result from an inability to effectively interact with a target protein. The ability of the H144Q mutant enzyme to complement is in accord with the finding that this mutant enzyme retains wild-type activity with two different protein substrates.

Table 1. Kinetics of DHFR refolding after mitochondrial import

	Time (min)			
Strain	Exp 1	Exp 2	Exp 3	Average
Wild-type	1.2	1.2	1.0	1.1
Wild-type + CsA	3.5	2.1	3.2	2.9
H144Q	1.7	1.5	1.1	1.4
H144Q + CsA	2.1	1.5	1.4	1.6

Exp, experiment.

FKBP12 Active-Site Mutants Are Protease Hypersensitive and Active with a Protein Substrate

We next tested whether our observations with cyclophilin active-site mutants also apply to FKBP12 active site mutants. Single amino acid substitutions in human FKBP12 (F36Y, D37V, and F99Y) have been described that perturb the active site and reduce prolyl isomerase activity in the in vitro peptide cleavage assay (Aldape *et al.*, 1992; Timerman *et al.*, 1995). Yeast and human FKBP12 share 54% identity and have nearly superimposable x-ray crystal structures (Heitman *et al.*, 1991a,b; Van Duyne *et al.*, 1991; Rotonda *et al.*, 1993). On the basis of this high degree of conservation, analogous active site mutations (F43Y, D44V, and F106Y) were introduced into yeast FKBP12. The wild-type and mutant FKBP12 proteins were tagged at their amino termini with hexahistidine, overexpressed in bacteria, and purified via Ni^{2+} affinity chromatography (see MATERIALS AND METHODS).

The activity of the mutant proteins was first assessed in the standard prolyl isomerase assay (Fischer *et al.*, 1984), as described above for the cyclophilin mutant enzymes. Wild-type FKBP12 shows a high activity in the protease-coupled assay (Figure 3A); isomerization is accelerated about 10-fold by 100 nM FKBP12. From the slope of the plot in Figure 3A a

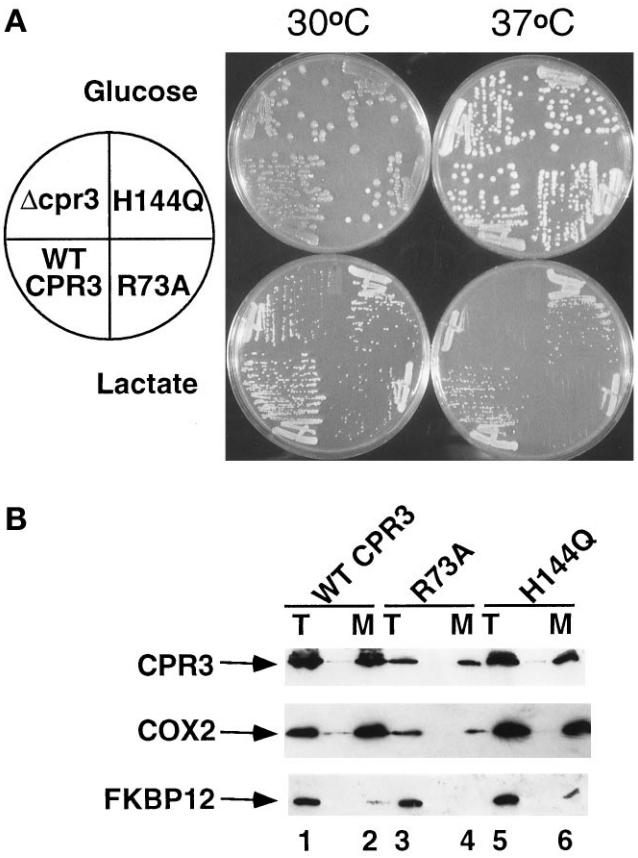

Figure 2. Mitochondrial cyclophilin active-site mutant functions in yeast. (A) CPR3 complementation assay. *cpr3* mutant strains (Eberhardt *et al.*, 1992) were transformed with a centromeric plasmid expressing wild-type Cpr3 or the R73A or H144Q mutant proteins and grown on solid glucose or lactate medium at 30°C or 37°C for 96 h. (B) Expression and localization of Cpr3 wild-type and mutant proteins. Total cell extracts (T) and mitochondrial fractions (M) were prepared as described (Yaffe, 1991) from the *cpr3::HIS3* strain JHY80–2B expressing the wild-type (lanes 1 and 2), R73A (lanes 3 and 4), or H144Q (lanes 5 and 6) Cpr3 protein tagged at the carboxyl terminus with the hemagglutinin (HA) epitope and analyzed by Western blot with anti-HA monoclonal antibodies to detect Cpr3p. Anti-COX2 antibodies (Molecular Probes, Eugene, OR) and FKBP12 antisera (Cardenas *et al.*, 1995) served to detect mitochondrial and cytoplasmic marker proteins, respectively.

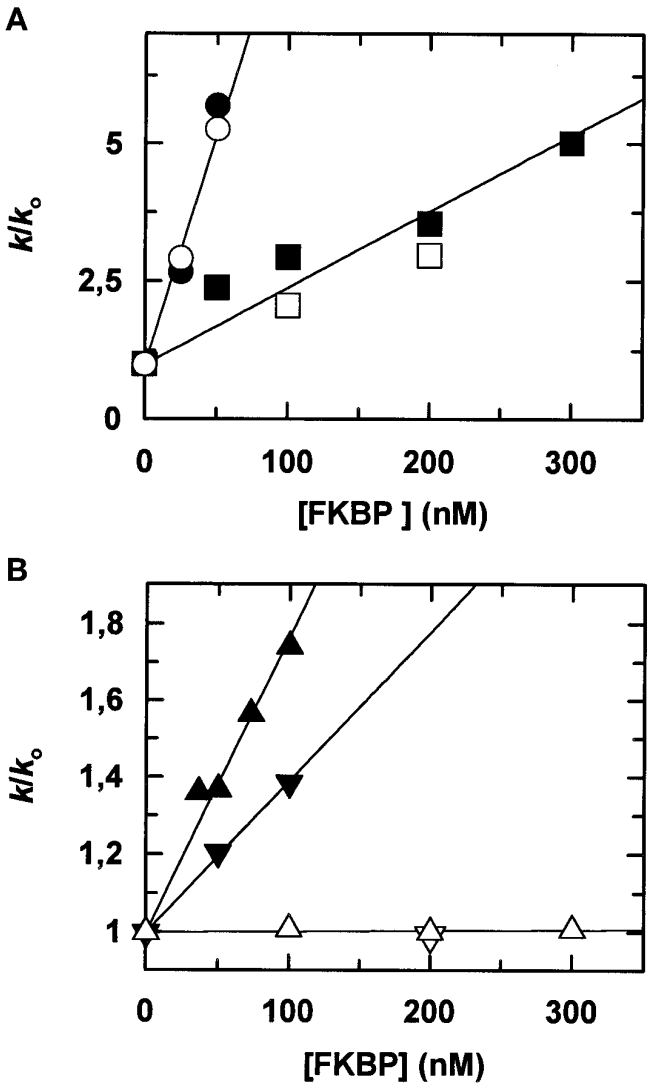

Figure 3. Activity of wild-type and FKBP12 mutants in the peptide-based prolyl isomerase assays. (A) Results for the wild-type protein (circles) and the F106Y mutant (squares). (B) Results for the F43Y (inverted triangles) and the D44V mutants (triangles). Activities measured by the protease-coupled assay are shown by the open symbols; activities measured by the protease-free peptide assays are shown by the solid symbols. The activities are given as k/k_0, where k and k_0 are the rate constants of isomerization in the presence and absence of these proteins, respectively. The activities were determined at 15°C as described in MATERIALS AND METHODS.

value of k_{cat}/K_m of 740,000 $M^{-1}\cdot s^{-1}$ can be determined. The F106Y mutation reduces prolyl isomerase activity about 10-fold, and the k_{cat}/K_m value for the F106Y variant is 85,000 $M^{-1}\cdot s^{-1}$, 11.5% the activity of wild-type FKBP12 (Figure 3A). In contrast, activity of the F43Y and the D44V mutant enzymes was barely detectable in the protease-linked activity assay (Figure 3B), and their k_{cat}/K_m values are smaller than 2000 and 3000 $M^{-1}\cdot s^{-1}$, respectively, which corresponds to <0.3 and <0.4% the activity of wild-type FKBP12 (Table 2).

To test whether the FKBP12 mutants might also be protease sensitive, we measured the activities of the wild-type and mutant FKBP12 enzymes in a protease-free peptide assay. The absorbance at 330 nm of the 4-nitroanilide moiety of the intact assay peptide decreases slightly upon *cis* to *trans* isomerization of the Phe-Pro imide bond. In the protease-free assay, a jump in the solvent conditions is used to shift the *trans*:*cis* equilibrium from 60:40 to 90:10. The concomitant change in absorbance is used to monitor the kinetics of Phe-Pro isomerization in the uncleaved assay peptide (Fischer, personal communication). The sensitivity of

Table 2. In vitro folding activities of wild-type and mutant FKBP12

FKBP	Protease-coupled assay (k_{cat}/K_m[$M^{-1}\cdot s^{-1}$])	Protease-free assay (k_{cat}/K_m[$M^{-1}\cdot s^{-1}$])	Protein folding assay (k_{cat}/K_m[$M^{-1}\cdot s^{-1}$])
Wild-type	740,000	810,000	49,000
F106Y	85,000	111,000	29,000
F43Y	<2,000	34,000	13,000
D44V	<3,000	46,000	51,000

the assay is limited because the decrease in absorbance is very small. It probably originates from a differential interaction between the 4-nitroanilide moiety and the phenylalanine residues in the *cis* and *trans* forms of the assay peptide. As expected, wild-type FKBP12 shows similar activities toward the assay peptide when measured either in the presence or the absence of the coupled protease. The apparent k_{cat}/K_m value determined in the absence of chymotrypsin is 810,000 $M^{-1}\cdot s^{-1}$ (Figure 3A), which is 10% larger than the k_{cat}/k_m of 740,000 $M^{-1}\cdot s^{-1}$ measured in the presence of the protease (Table 2). This difference is expected because in the irreversible protease-coupled assay the measured activity is determined solely by the rate of the *cis* to *trans* reaction, whereas in the reversible protease-free assay the rates of both the *cis* to *trans* and the *trans* to *cis* reactions contribute to the activity measured. Although the activity of the F106Y mutant enzyme is 10-fold reduced relative to the wild-type protein, this mutant is resistant to proteolysis in the assay and yields nearly identical k_{cat}/K_m values of 85,000 $M^{-1}\cdot s^{-1}$ and 111,000 $M^{-1}\cdot s^{-1}$ in the presence and absence of protease, respectively. However, the F43Y and the D44V mutants, which were virtually inactive in the protease-coupled assay, exhibit k_{cat}/K_m values of 34,000 $M^{-1}\cdot s^{-1}$ and 46,000 $M^{-1}\cdot s^{-1}$ in the protease-free assay (Figure 3B and Table 2). This indicates that their lack of activity in the presence of the coupled protease is caused by an inactivation by proteolytic degradation. It also indicates that these two mutants show a low but significant residual activity of ~5% toward the Phe-Pro bond in the peptide substrate.

We next measured the prolyl isomerase activity of the mutant FKBPs in a protein folding assay that uses as substrate the reduced and carboxymethylated variant of ribonuclease T1 RCM-(S54G/P55N)-RNase T1, as described above for the cyclophilin mutant enzymes. In the absence of enzyme, this folding reaction shows a time constant of 570 s, and 200 nM wild-type FKBP12 accelerates the reaction sevenfold. A k_{cat}/K_m value of 49,000 $M^{-1}\cdot s^{-1}$ can be calculated from the increase in the folding rate as a function of the FKBP12 concentration (Figure 4). For folding protein substrates the k_{cat}/K_m values are generally smaller than for peptides because the prolyl bonds are partially shielded in folding intermediates (Schmid, 1993) and because nonproductive binding may occur. The D44V mutation did not affect the prolyl isomerase activity of FKBP12 in the folding assay, and virtually the same k_{cat}/K_m value of 51,000 $M^{-1}\cdot s^{-1}$ was observed as with the wild-type enzyme (Figure 4 and Table 2). This is in marked contrast to both the protease-linked and the protease-free peptide assays (Figure 3B and Table 2), in which the relative activities of the D44V mutant enzyme were less than 1 and 5%, respectively. The F106Y and the F43Y mutants were 60% and 30%, respectively, as active as wild-type FKBP12 in the protein folding assay. Again, the relative activities of these two mutants toward a protein substrate were much higher than their relative activities toward a peptide substrate in the protease-free assays (Table 2).

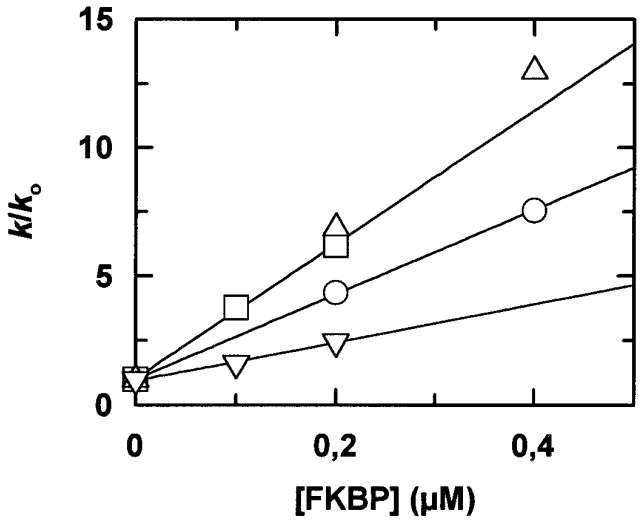

Figure 4. Wild-type and FKBP12 mutants catalyze ribonuclease T1 refolding. The kinetics of refolding of 0.3 μM RCM-(S54G/P55N)-RNase T1 were measured in the absence and in the presence of increasing concentrations of the wild-type and the mutant forms of FKBP12. The measured catalytic activities in folding are shown as a function of the concentration of wild-type FKBP12 (□), the F106Y (○), the D44V (△), and the F43Y (▽) mutants. The activities are shown as k/k_0, where k and k_0 are the rate constants of folding of RCM-(S54G/P55N)-RNase T1 in the presence and absence of the various FKBP12 proteins, respectively. The folding kinetics were measured at 15°C in 0.1 M Tris-HCl and 2.0 M NaCl by the increase in fluorescence at 320 nm.

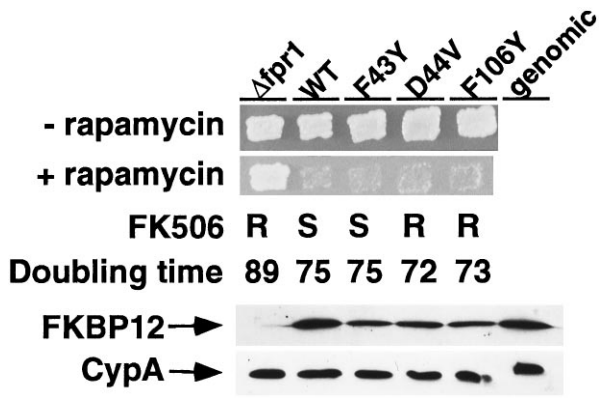

Figure 5. Active-site FKBP12 mutants are functional in vivo. Drug resistance. Strains JHY2–1c (*fpr1*; Heitman *et al.*, 1991a,b) and CHY516 (*vph6 fpr1*) were transformed with a low-copy-number centromeric plasmid bearing the wild-type *FPR1* gene (WT), the F43Y, D44V, or F106Y mutant genes, or no *FPR1* gene (Δ*fpr1*). Transformants were grown on synthetic medium lacking leucine without (− rapamycin) or with 0.1 μg/ml rapamycin (+ rapamycin), or with 1 μg/ml FK506. FK506 sensitivity was assayed in an *vph6 fpr1* mutant strain in which calcineurin is essential for growth (Hemenway *et al.*, 1995); drug-resistant growth and drug-sensitive growth are indicated by R and S, respectively. Growth rate. The doubling times (expressed in minutes) of the transformants were determined by monitoring the OD_{600} of liquid cultures grown at 30°C. Protein expression. Protein extracts were analyzed by Western blot with an anti-FKBP12 polyclonal antisera (Cardenas *et al.*, 1995).

The activities of all FKBP12 forms in the three assays are compared in Table 2. We conclude that all of these FKBP12 active-site mutants retain near wild-type levels of activity with a protein substrate and that the F43Y and D44V mutations render the enzyme protease hypersensitive and thus artifactually inactive in the protease-coupled peptide isomerization assay.

FKBP12 Active-Site Mutants Are Functional In Vivo

We next tested whether these FKBP12 active-site mutants provide FKBP12 functions in vivo. Yeast mutants lacking FKBP12 (encoded by the *FPR1* gene) are viable but exhibit slow growth, with a doubling time increased by 15–30% compared with an isogenic wild-type strain (Heitman *et al.*, 1991a,b). In addition, FKBP12 is the cellular receptor for the macrolides FK506 and rapamycin; thus, *fpr1* mutants are resistant to rapamycin (Heitman *et al.*, 1991a,b) and also to FK506 under conditions, such as cation stress, or in mutant strains (*vph6*) in which the target of FKBP12-FK506, calcineurin, is essential (Nakamura *et al.*, 1993; Breuder *et al.*, 1994; Hemenway *et al.*, 1995).

The genes encoding wild-type and the mutant FKBP12 proteins were expressed from a low-copy-number centromeric plasmid and tested for complementation of *fpr1* mutant phenotypes (see MATERIALS AND METHODS). By Western blot, the F43Y, D44V, and F106Y mutant proteins were expressed at a slightly reduced level compared with wild-type FKBP12 expressed from a centromeric plasmid or the chromosome (Figure 5). In accord with previous studies (Aldape *et al.*, 1992; Lorenz and Heitman, 1995), wild-type FKBP12 and the F43Y, D44V, and F106Y mutant proteins restored sensitivity to rapamycin, indicating sufficient rapamycin binding to FKBP12 and FKBP12-rapamycin binding to TOR1/TOR2 to inhibit yeast growth. Wild-type and the F43Y mutant proteins restored FK506 sensitivity (Figure 5) and thus bind FK506 and calcineurin, whereas the D44V and F106Y mutant enzymes did not, in accord with previous findings that the corresponding residues of human FKBP12, D37 and F99, are critical for FK506 binding (DeCenzo *et al.*, 1996). More importantly, wild-type FKBP12 and the F43Y, D44V, and F106Y mutant proteins all complemented and restored normal growth rate in yeast FKBP12 null mutant cells (Figure 5). That these FKBP12 mutant enzymes are active in vivo is in full accord with our finding that all retain activity with a protein substrate in vitro.

Heterologous FKBP Prolyl Isomerases Lack FKBP12 In Vivo Function

As a complementary approach to assess the importance of the prolyl isomerase activity of FKBP12 for its functions in yeast, we tested whether human FKBP12 or yeast FKBP13 lacking its signal sequence could complement a yeast FKBP12 null mutation. To ensure that each enzyme was expressed at a similar level, the 5′ and 3′ untranslated regulatory regions of the yeast FKBP12 gene *FPR1* were fused to the start and stop codons of each open reading frame by PCR overlap (see MATERIALS AND METHODS). The resulting chimeric genes express human FKBP12 and yeast FKBP13 lacking the signal sequence. Both human FKBP12 and yeast FKBP13 lacking the signal sequence complemented FKBP12 mutant strains and restored sensitivity to rapamycin (Figure 6). In contrast, human FKBP12 partially restored sensitivity to FK506, whereas the FKBP13 prolyl isomerase did not (Figure 6), consistent with previous findings that FKBP12 surface residues required to inhibit calcineurin are altered in FKBP13 (Aldape *et al.*, 1992). Most importantly, human FKBP12 and the FKBP13 prolyl isomerase domains did not complement the growth defect of yeast FKBP12 mutants. The heterologous FKBP domains were expressed, based on restoration of drug sensitivities, as described above. Thus, expression of an active FKBP prolyl isomerase is not sufficient to provide FKBP12 function(s) required for normal growth.

To address FKBP12 structural features required for in vivo function, chimeras were constructed between yeast and human FKBP12. As shown in Figure 6, the amino-terminal two-thirds of human FKBP12 were fused to the carboxyl-terminal one-third of yeast

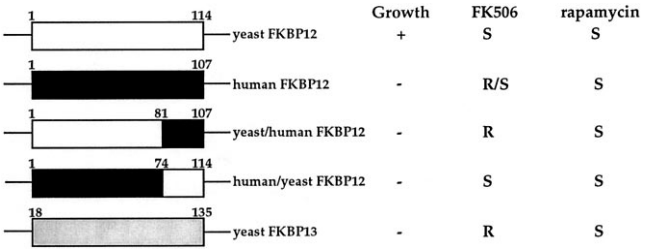

Figure 6. Expression of heterologous FKBP domains in yeast FKBP12 mutants. The 5′ and 3′ regulatory regions of the yeast FKBP12 gene were fused to the open reading frames from human FKBP12, yeast FKBP13 lacking the secretory signal sequence, and yeast-human and human-yeast chimeric FKBP12 genes. The resulting genes were expressed from a CEN plasmid in *vph6 fpr1* and *fpr1* mutant strains CHY516 and JHY2–1c, as in Figure 1, to assay complementation and restoration of drug sensitivity. S, R/S, and R indicate sensitivity, partial sensitivity, and resistance, respectively. Complementation of the growth defect of FKBP12 mutant strain JHY2–1c by heterologous FKBP domains was assessed on solid medium and by determining doubling times of the transformants grown in liquid culture (YPD medium) at 30°C. +, complementation; −, no complementation.

FKBP12 (human-yeast FKBP12), and the amino-terminal two-thirds of yeast FKBP12 were fused to the carboxyl-terminal one-third of human FKBP12 (yeast-human FKBP12). As above, the chimeric genes contained the 5′ and 3′ regulatory regions of the yeast FKBP12 gene. Both human-yeast FKBP12 and yeast-human FKBP12 restored rapamycin sensitivity, and the human-yeast FKBP12 hybrid restored FK506 sensitivity, but neither complemented the growth defect of FKBP12 mutant strains (Figure 6). We conclude that additional structural features of yeast FKBP12 important for in vivo function lie in both the amino-terminal two-thirds and the carboxyl-terminal one-third of the protein.

DISCUSSION

Our original aim was to test whether FKBP and cylophilin prolyl isomerase activity was required for in vivo function. The approach was to introduce active-site mutations previously shown to result in a loss of in vitro isomerase activity and to test whether these mutants would complement FKBP12 and cyclophilin null mutations when expressed in yeast. Somewhat to our surprise, we found that these active-site mutants were, with one exception, fully functional in vivo. This finding prompted us to reexamine the original premise that these active-site mutations result in a loss of isomerase activity. In a reciprocal approach, we tested whether heterologous FKBP domains can restore function in the FKBP12 null strain. We found that different FKBP prolyl isomerase domains, derived from human FKBP12 and yeast FKBP13, do not provide FKBP12 biological function in yeast. Hence, FKBP prolyl isomerase activity alone is not sufficient for FKBP12 function in vivo.

To reexamine the active-site mutations in mitochondrial cyclophilin and FKBP12, we measured the isomerase activity of mitochondrial cyclophilin (wild-type and R73A and H144Q active-site mutants) and FKBP12 (wild-type and F43Y, D44V, and F106Y active-site mutants) in the standard peptide assay for prolyl isomerase activity. The mutants of both the Cpr3 and the FKBP12 enzyme had dramatically reduced activity in this chymotrypsin-coupled peptide assay. In contrast, by using a protease-free peptide assay, we found that the R73A and H144Q Cpr3 mutant enzymes and also the F43Y and D44V FKBP12 mutant enzymes had demonstrable levels of activity.

The H144Q active-site mutant is almost as active as the wild-type Cpr3 enzyme in an assay that measured folding of murine DHFR in purified mitochondria expressing wild-type or mutant Cpr3 enzymes. Furthermore, all of the Cpr3 (wild-type or active-site mutant) and FKBP12 (wild-type or active-site mutant) enzymes catalyzed the in vitro refolding of RCM-(S54G/P55N)-ribonuclease T1, which is limited in rate by the *trans* to *cis* isomerization of the Tyr^{38}-Pro^{39} peptidyl-prolyl bond. This folding reaction can be catalyzed by many different cyclophilins and FKBPs (Kiefhaber *et al.*, 1990a,b; Tropschug *et al.*, 1990; Schönbrunner *et al.*, 1991; Schmid, 1993; Mayr *et al.*, 1996; Scholz *et al.*, 1997a). The R73A and the H144Q active-site mutants of the mitochondrial cyclophilin Cpr3 showed about 70% of the activity of the wild-type protein in the catalysis of this folding reaction. Similarly, the three active-site mutants of FKBP12 showed between 30 and 100% of the folding activity of the wild-type protein. These findings indicate that it will be necessary to test candidate active-site mutants for FKBPs, cyclophilins, and parvulins by using protein-folding assays with ribonuclease T1 or other substrates.

Why are these active-site mutants inactive in an in vitro protease-coupled peptide assay but retain activity in a protease-free peptide assay and with two protein substrates? First, the R73A and H144Q cyclophilin mutant enzymes and the F43Y and D44V FKBP12 mutant enzymes are markedly sensitive to proteolysis by chymotrypsin, possibly as a consequence of conformational changes or decreased stability, and thus are rapidly destroyed in the protease-coupled peptide assay. Even in protease-independent assays, however, the active-site mutants were still 3- to 20-fold less active than with a protein substrate in the folding assays (Table 2). One possible explanation is that large substrates can use additional contacts to the enzyme and that these contacts mitigate the deleterious effects of active site mutations. Another possible explanation for the higher activity in the protein folding assay compared with the peptide assay is that the protein substrate may preorganize the prolyl-peptide

bond in a conformation more favorable for catalysis (Fischer *et al.*, 1993).

Our findings are relevant to earlier studies that addressed the roles of FKBP and cyclophilin prolyl isomerase activity in other systems. Human FKBP12 is a subunit of the ryanodine and inositol 1,4,5-trisphosphate (IP_3) receptors and is required for proper Ca^{2+} channel activity (Brillantes *et al.*, 1994; Cameron *et al.*, 1995). The finding that FKBP12 mutants with reduced prolyl isomerase activity in the in vitro peptide cleavage assay support channel function has been interpreted to mean that prolyl isomerase activity is not required for this FKBP12 function (Timerman *et al.*, 1995). Alternatively, these FKBP12 mutant enzymes may retain activity toward this large substrate, as our findings clearly demonstrate is the case with ribonuclease T1 as a substrate.

The study identifying the novel cyclophilin RanBP2 is worth revisiting (Ferreira *et al.*, 1996). The active-site mutations used in the RanBP2 study are analogous to those studied herein. Because the RanBP2 active-site mutants prevented binding and alteration of opsin by the RanBP2, it was suggested that prolyl isomerization of opsin by RanBP2 cyclophilin was critical for function (Ferreira *et al.*, 1996). Our findings that the analogous active-site mutants do not effect the ability of mitochondrial cyclophilin to refold ribonuclease T1 argue that these mutations may instead perturb some other feature of the opsin–cyclophilin complex. We note that while the R73A Cpr3 mutant protein folds ribonuclease T1 in vitro, this mutant failed to provide Cpr3 function in yeast, and hence the residues within the active sites of both yeast mitochondrial cyclophilin and the RanBP2 cyclophilin are clearly implicated in function. The defects of these mutant enzymes could result from loss of activity for specific substrates or from a loss of another function of their active-site binding pockets, such as the ability to form stable protein–protein complexes with target molecules.

R55 (analogous to R73 in Cpr3) was originally implicated as a critical active-site residue of human cyclophilin A by the mutagenic and biochemical studies of Zydowsky *et al.* (1992). In addition, the x-ray crystal structure of human cyclophilin A in complex with the tetrapeptide substrate Ala-Ala-Pro-Phe revealed the guanidine group of Arg^{55} is hydrogen bonded to the carbonyl oxygen of the substrate proline (Zhao and Ke, 1996). On the basis of this structure, it was proposed that during catalysis the guanidine nitrogen of Arg^{55} might hydrogen bond with the peptide nitrogen, weakening the partial double-bond properties of the peptide bond and facilitating catalysis. Although we cannot exclude that Arg^{73} might play some role in catalyzing *cis* to *trans* isomerization of peptide substrates, our finding that Arg^{73} of mitochondrial cyclophilin can be replaced by alanine without loss of catalytic activity excludes an obligate role for the Arg^{73} side chain in catalysis of *trans* to *cis* isomerization in ribonuclease T1.

What is the role of the enzymatic activity of FKBP12 and mitochondrial cyclophilin for biological function? Recent studies of ninaA and of human cyclophilin A with their interaction partners rhodopsin and HIV-1 GAG protein, respectively, reveal that these interactions may involve stoichiometric protein–protein complexes rather than catalytic enzyme–substrate interactions (Baker *et al.*, 1994; Gamble *et al.*, 1996; Luban, 1996). These abundant proteins may have originally served a catalytic function for partner proteins but later in evolution been conscripted to serve as subunits independent of catalytic activity. Other functions may require enzymatic activity. Prolyl isomerases could still participate in protein folding, either by isomerizing a limited number of substrates or as chaperones, independent of prolyl isomerase activity. A recent report, however, that human cyclophilin A exhibits chaperone activity has been controversial (Freskgard *et al.*, 1992; Kern *et al.*, 1994). Alternatively, prolyl isomerases might not fold proteins but rather serve specialized roles yet to be revealed. Finally, because cyclophilins and FKBPs serve as the intracellular receptors for natural product immunosuppressive ligands and mediate the ability of these compounds to inhibit target proteins, the binding pockets of cyclophilin and FKBP may regulate the function of other proteins in response to binding of as yet unknown endogenous ligands. Further insights into the roles of the prolyl isomerases in yeast and other organisms will require the identification of additional target proteins, studies that are now in progress.

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